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14. ABSTRACT

Wnts are lipid-modified secreted glycoproteins that regulate diverse biological processes. I have shown that Wnt5a, which functions in non-canonical Wnt signalling, promoted proliferation, cell survival and network formation on endothelial cells. In new experiments, Wnt5a induced non-canonical Wnt signalling in endothelial cells, as measured by Dishevelled and ERK1/2 phosphorylation, as well as inhibited canonical Wnt signaling. Using shRNA technology to target Wnt5a expression, both endothelial cell network formation and migration were inhibited. A DNA array screening for Wnt5a-regulated genes in cultured cells identified several encoding angiogenic regulators, and Tie-2, a receptor for angiopoietins, was validated as a new target gene of non-canonical Wnt signaling in human endothelial cells. So far, non-canonical Wnt signaling had unknown functions on endothelial cell biology. Experimental findings from this project highlight the importance of non-canonical Wnt signaling on angiogenesis.

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Introduction

The Wnt family of proteins comprises a group of secreted, lipid-modified glycoproteins that play crucial roles in the regulation of developmental patterning, cell proliferation, differentiation, polarity and morphogenetic movement (Logan and Nusse, 2004). Wnts trigger intracellular responses through various signalling pathways, referred to as canonical or non-canonical, utilizing the Frizzled family of receptors (Veeman *et al.*, 2003). While canonical Wnts signal by blocking degradation of cytosolic β-catenin and hence activating β-catenin/TCF transcriptional responses, non-canonical Wnts are thought to activate the Wnt/Ca^{++}, Wnt/planar cell polarity (PCP) or other less well-defined pathways. However, a consistent response to non-canonical Wnt signals is the phosphorylation of Dishevelled (Dvl) proteins (Gonzalez-Sancho *et al.*, 2004). Wnt5a has been shown to function in non-canonical Wnt signalling in several systems. For example, expression of Wnt5a in zebrafish or Xenopus embryos can stimulate intracellular Ca^{2+} fluxes (Slusarski *et al.*, 1997), and Wnt5a regulates morphogenetic movements during gastrulation independently of canonical signalling (Kilian *et al.*, 2003). Consistent with non-canonical signalling, ectopic over-expression of Wnt5a in human primary endothelial cells does not stabilize cytosolic ß-catenin nor activate ß-catenin/TCF-mediated transcription (Wright *et al.*, 1999; Masckauchan *et al.*, 2005).

Wnt5a is essential for proper skeletal development as nullizygous mice exhibit perinatal lethality and appendicular structures outgrowing from the primary body axis fail to extend (Yamaguchi *et al.*, 1999). This defect in morphogenesis is associated with decreased cell proliferation in tissues crucial to outgrowing structures. In addition, Wnt5a may also participate in hematopoietic stem cell fate decisions (Murdoch *et al.*, 2003). Wnt5a can also negatively influence proliferation, for instance in B cells (Liang *et al.*, 2003). Increased expression of Wnt5a has been reported in malignant melanoma, primary human breast cancer, and colon cancer, as well as cancer from other tissues, suggesting a pathological effect on tumorigenesis that may be either positive or negative (Iozzo *et al.*, 1995; Lejeune *et al.*, 1995; Jonsson *et al.*, 2002; Weeraratna *et al.*, 2002; Dejmek *et al.*, 2005).

The signalling cascades that are activated by Wnt5a to regulate proliferation are not well defined. Wnt5a can interact with Wnt receptors Frizzled-2 (Slusarski *et al.*, 1997), Frizzled-4 (Chen *et al.*, 2003), Frizzled-5 (Sen *et al.*, 2001) and Frizzled-7 (Umbhauer *et al.*, 2000). In different systems, Wnt5a has been shown to activate protein kinase C (Weeraratna *et al.*, 2002), C-Jun N-terminal kinase (Yamanaka *et al.*, 2002) and induce phosphorylation of Dishevelled (Gonzalez-Sancho *et al.*, 2004; Schulte *et al.*, 2005). Signal activation by Wnt5a can also negatively influence the Wnt/ß-catenin pathway by several different mechanisms (Saneyoshi *et al.*, 2002; Ishitani *et al.*, 2003; Topol *et al.*, 2003).

Expression of Wnt5a has been reported in human endothelial cells (Wright *et al.*, 1999; Masckauchan *et al.*, 2005), yet its function in endothelium has remained largely unknown. As Wnt5a is a known inducer of non-canonical signaling, I ectopically expressed Wnt5a in endothelial cells in order to ask whether non-canonical

signaling promotes angiogenesis. Additionally, a large amount of evidence links non-canonical Wnts with development of various forms of cancer, including breast cancer, its function and regulation is yet mostly unexplored. In consequence, new findings in this area can be a contribution of great interest to cancer research.

Report Body

Wnt5a induces Dishevelled phosphorylation, ERK phosphorylation and inhibits canonical Wnt signalling in human endothelial cells

Wnt5a signalling is known to induce phosphorylation of Dishevelled in several cell types, and this can be monitored by a mobility shift of Dvl proteins on Western blots (Gonzalez-Sancho *et al.*, 2004; Schulte *et al.*, 2005). This effect is independent of the canonical Wnt signalling co-receptors LRP5 and LRP6 and is thus a manifestation of non-canonical Wnt signalling (Gonzalez-Sancho *et al.*, 2004). Analysis of endothelial cells treated with purified Wnt5a protein led to demonstrably increased phosphorylation of Dvl2 and Dvl3 (Fig. 1A). To further analyze signalling downstream of Wnt5a in human endothelial cells, I used a HA-tagged Wnt5a-expressing adenovirus vector (Ad-Wnt5a) that allows Wnt5a production in cultured primary endothelial cells (Fig. 1B). Ectopically expressing Wnt5a also caused an increase in Dishevelled phosphorylation (not shown). Expression of Wnt5a via adenovirus also led to induction of ERK1/2 phosphorylation, as evaluated by Western blot (Fig. 1C). Thus, Wnt5a induces Dvl phosphorylation and activates ERK1/2 in primary endothelial cells.

Wnt5a non-canonical signalling has been shown to antagonize the canonical ß-catenin/TCF pathway (Topol *et al.*, 2003). I analysed whether this was also the case for human endothelial cells. HUVEC were co-transfected with plasmids encoding either Wnt5a, ß-cateninS37A (a stabilized form of ß-catenin) or LacZ genes and TCF/Lef reporter constructs. Included in the transfections was a renilla-luciferase plasmid to normalize readings for transfection efficiency. Twenty-four hours after transfection, luciferase activity showed that increasing amounts of Wnt5a antagonized ß-cateninS37A-mediated activation of the TCF/Lef reporter in human endothelial cells, in a dose-responsive manner (Fig. 1D). Thus, Wnt5a signals via non-canonical pathways in HUVEC, as measured by suppression of canonical Wnt signalling.

Non-canonical Wnt signalling acts via several distinct pathways, including Ca++, PKC, JNK, and pathways that antagonize canonical Wnt signalling. Non-canonical Wnt signalling also leads to hyper-phosphorylation of the intracellular protein dishevelled (Gonzalez-Sancho *et al.*, 2004). I found that Wnt5a addition to human primary endothelial cells induces phosphorylation of Dvl-2 and Dvl-3. Since I previously established that Wnt5a does not activate Wnt/ß-catenin signalling in endothelial cells (Masckauchan *et al.*, 2005), I can conclude that Wnt5a acts on endothelial cells in a non-canonical manner. In the past, I was unable to detect significant changes in neither NFAT, PKC, nor JNK signalling in response to Wnt5a expression in endothelial cells (unpublished data). However, Wnt5a promoted ERK1/2 phosphorylation in endothelial cells. Previous studies showed that Wnt5a promotes ERK1/2 phosphorylation in serum-starved MC3T3-E1 cells, where Wnt5a was able to protect cells from apoptosis via ERK signalling (Almeida *et al.*, 2005). Wnt3a, a

canonical signalling Wnt, can also promote ERK1/2 phosphorylation, and this signal was proposed to mediate proliferation of NIH3T3 cells (Yun *et al.*, 2005). Concomitant with this, I have previously shown that increased levels of cytosolic ß-catenin in endothelial cells can lead to cell proliferation and survival (Masckauchan *et al.*, 2005). In recent studies, I have found that Wnt5a expression in endothelial cells blocks canonical Wnt signalling, a known function of Wnt5a in other cell types (Topol *et al.*, 2003).

Gene expression analysis in response to Wnt5a showed regulation of a mostly distinct set of genes than that found with Wnt-1, a Wnt that stimulates Wnt/ß-catenin signaling in endothelial cells (Masckauchan *et al.*, 2005). This observation supports the hypothesis of an antagonistic role for non-canonical Wnt signalling on canonical signalling in endothelial cells. The existence of a common pathway like ERK1/2 activated by both canonical and non-canonical Wnt signalling, the antagonistic role of Wnt5a on canonical Wnt signalling, and the fact that the genes regulated by either of these pathways differ in endothelial cells, point to the notion that Wnt5a has at least one other mechanism of signal transduction in angiogenesis.

Wnt5a promotes endothelial network formation and endothelial cell migration

The ability of endothelial cells to form a network of capillaries after they migrate from a pre-existing blood vessel is a crucial property in both normal and tumor angiogenesis. I further analyzed the ability of HUVEC cultured on top of extracellular matrix components to form a network-like structure when they were either overexpressing Wnt5a or when endogenous expression of this gene was decreased by shRNA. HUVEC were programmed to overexpress HA-tagged Wnt5a via retroviral vectors (Wnt5a-HA-HUVEC) and used in a matrigel assay for cord formation. After 18 hours of growth on matrigel, Wnt5a-HA HUVEC formed more extensive networks than control, HA-expressing HUVEC (Fig. 2A, upper panel). Although the number and length of branches were similar, branching point size was increased by 81.4 %, with $p<0.001$ in Wnt5a–HA expressing cells compared to –HA controls. Analyzing 6 different candidate target sequences to silence the human Wnt5a gene, I found one that successfully silences this gene, chose it to prepare a retrovirus encoding for shRNA-Wnt5a, infect HUVEC with this virus and select them with puromycin. Consistent with the previous observation that Wnt5a induced network formation, I observed reduced networks of endothelial cells when the same experiment was done using retrovirally infected cells with shRNA-mediated reduction of Wnt5a, as compared to control (Fig. 2A, lower panel). In this case, branching point size decreased by 32 %, with $p<0.001$ in Wnt5a-expression reduced cells compared to control cells. Reduction of Wnt5a expression via shRNA also caused reduced migration of endothelial cells into wounded areas of HUVEC monolayers, as monitored 10 hours after wounding (Fig. 2B). Cell front separation was reduced by only 33 ± 4.5 % in cells with reduced Wnt5a expression, while control shRNA cells decreased front separation by 54 ± 5 %. However, enhanced cell motility was not observed when Wnt5a was ectopically overexpressed.

FIGURE 1

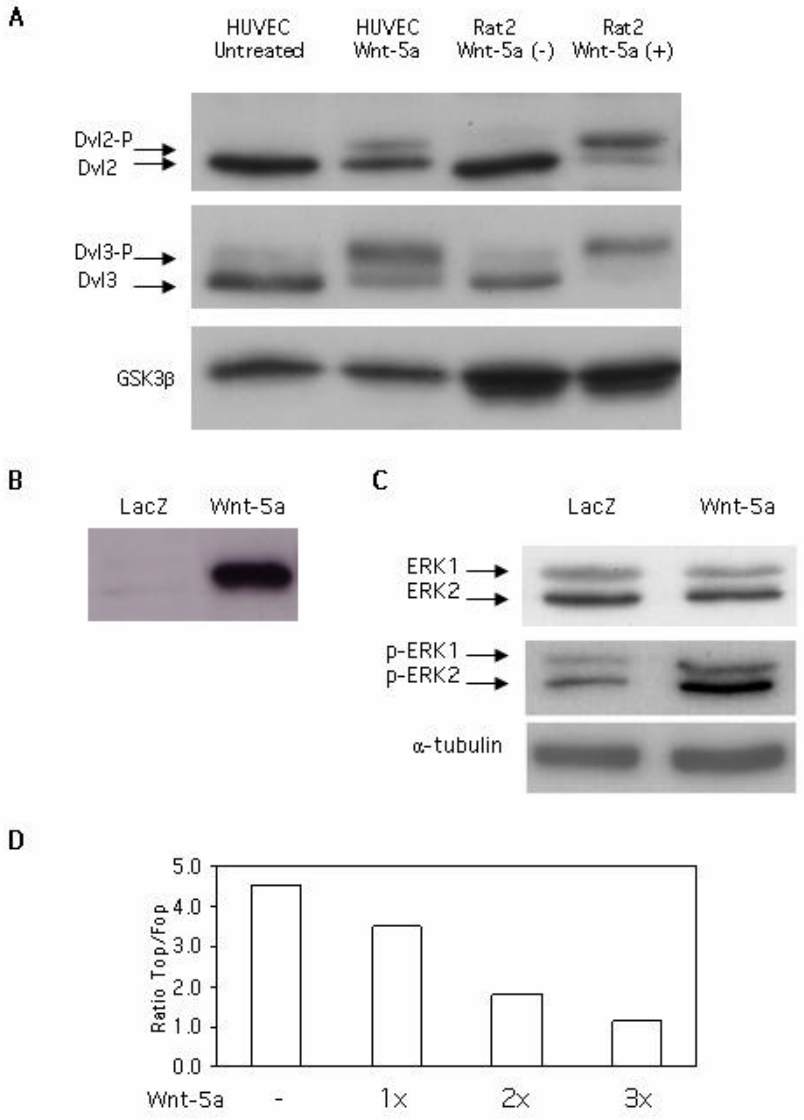

Figure 1

Wnt5a induces Dishevelled and ERK1/2 phosphorylation, and antagonizes canonical Wnt signalling in human endothelial cells. (A) Mobility shift assay for Dishevelled phosphorylation. HUVEC were treated with purified Wnt5a protein (75 ng/mL) for 2 hrs and lysates were prepared. Western blot to detect Dishevelled2 or Dishevelled3 was carried out using GSK3β as loading control. Rat2 cells overexpressing Wnt5a or not were used as a (+) and (-) controls, respectively. (B) Western blot showing ectopic expression of adenovirus-infected HUVEC overexpressing control LacZ gene or Wnt5a-HA. (C) Western blot for total and phospho-ERK1/2. HUVEC were infected to overexpress Wnt5a or control (LacZ) gene, and lysates were prepared 48 hrs later. (D) Wnt-5a antagonizes canonical signaling in endothelial cells. Tcf-responsive constructs SuperTop- or control SuperFop-luciferase were co-transfected into HUVEC with constant amounts (0.34 ug/well) of wild type β-catenin plasmid in presence of increasing amounts in ug (no Wnt5a, 1-, 2- or 3-fold) of Wnt5a plasmid. Empty vector plasmid was used to transfect cells with equal total amount of plasmid.

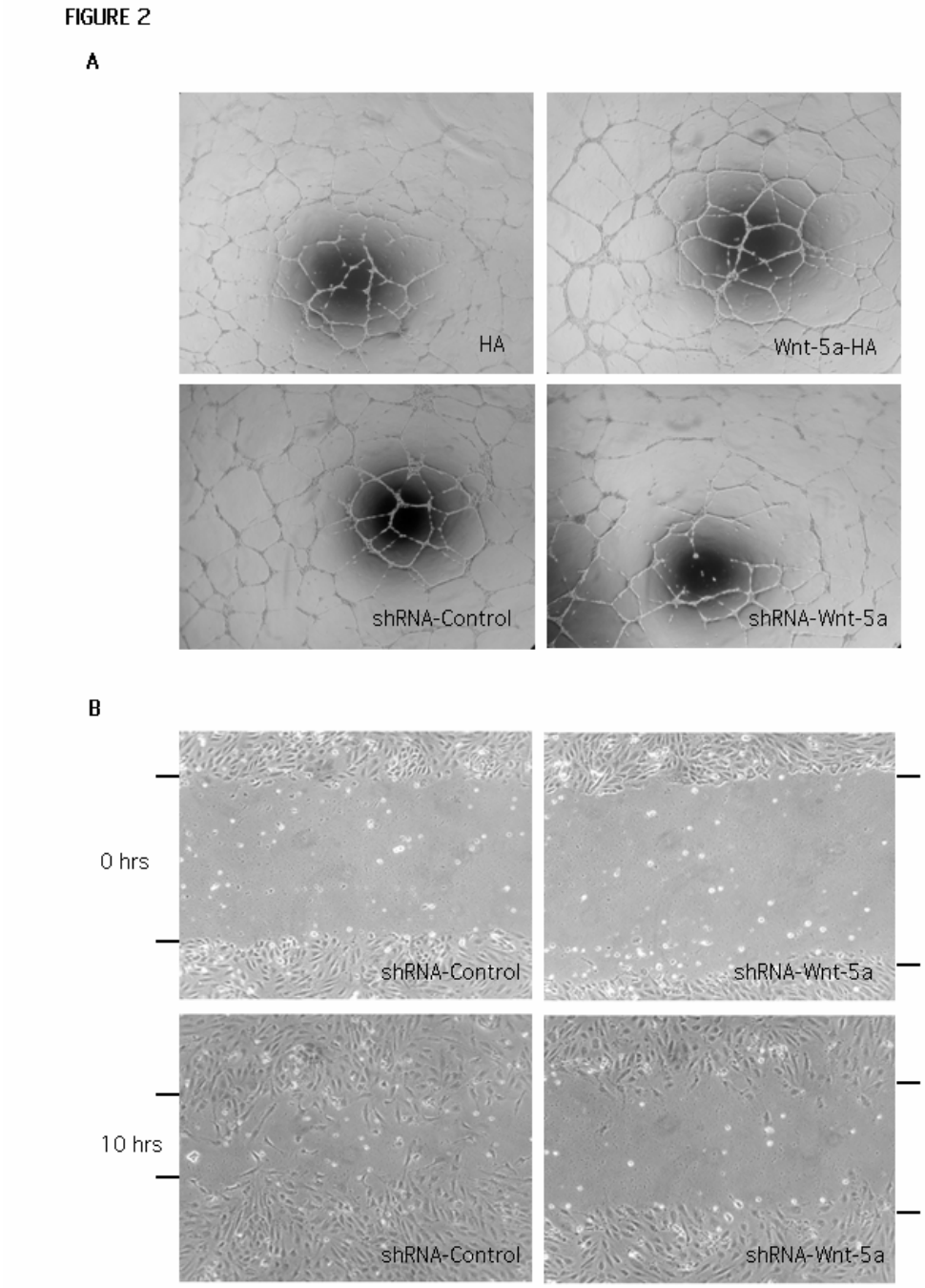

Figure 2

Wnt5a participates in capillary-like networks of endothelial cells and endothelial cell migration. (A) Retroviral-infected HUVEC either overexpressing Wnt5a-HA, compared to control (HA), showed enhanced formation of networks (top pictures). HUVEC overexpressing shRNA to target Wnt5a gene expression showed reduced ability to form networks when compared to control shRNA cells. (B) Retroviral infected HUVEC overexpressing shRNA targeting Wnt5a gene expression showed reduced ability to migrate, measured 10 hours after a wound was made on a confluent plate, when compared to control cells. A representative experiment is shown. Black bars indicate cell front.

Non-canonical Wnt signalling induces Tie-2 expression in endothelial cells.

To better understand the potential mechanisms by which wnt5a signalling promoted endothelial cell proliferation, survival and network formation, I screened for genes differentially regulated by wnt5a in endothelial cells. HUVEC were infected with either Ad-Wnt5a or Ad-LacZ and ectopic expression of Wnt5a was confirmed by western blot. The expression profile of cells over-expressing Wnt5a was compared to Wnt-1 and lacz (control) overexpressing cells by conducting DNA microarray analysis as described (Fig. 3). For the current study, the most interesting was the largest clade, in which genes were highly expressed in Wnt5a but low or intermediate in LacZ and Wnt1. The identities and fold regulation of some of these genes, which are predicted to be direct or indirect downstream targets activated by Wnt5a are listed on Table I. Two differentially expressed genes, encoding the angiogenic regulators MMP-1 and Tie2 (or TEK), were selected for further analysis in order to confirm they were regulated by overexpression of wnt5a in primary endothelial cells. MMP-1 was previously confirmed as a Wnt5a target gene by PCR, Western blot and an ELISA analysis in culture media collected from HMVEC, (see Annual Report Year 2). In the last year, my work focused on validating Tie2 as a gene target for non-canonical Wnt signalling in human endothelial cells. There were other candidate genes in the DNA array analysis (a selection of the most relevant target genes found are listed on Table I), which were analysed by PCR with specific primers but could not be validated.

Tie-2, a receptor of angiopoietin 1 and 2 and a key regulator of angiogenesis, was shown to be moderately upregulated in these cells, as confirmed by RT-PCR and Western blot analysis (Figs. 4A and B, respectively). As evaluated by Western blot, Wnt5a increased Tie-2 expression by 5.9-fold and 4.5-fold when compared to LacZ and ß-cateninS37A, respectively (Fig. 4B). Thus, Wnt5a signalling leads endothelial cells to increased production of known angiogenic regulators like MMP-1 and Tie2, and this may aid in the pro-angiogenic activities of Wnt5a.

FIGURE 3

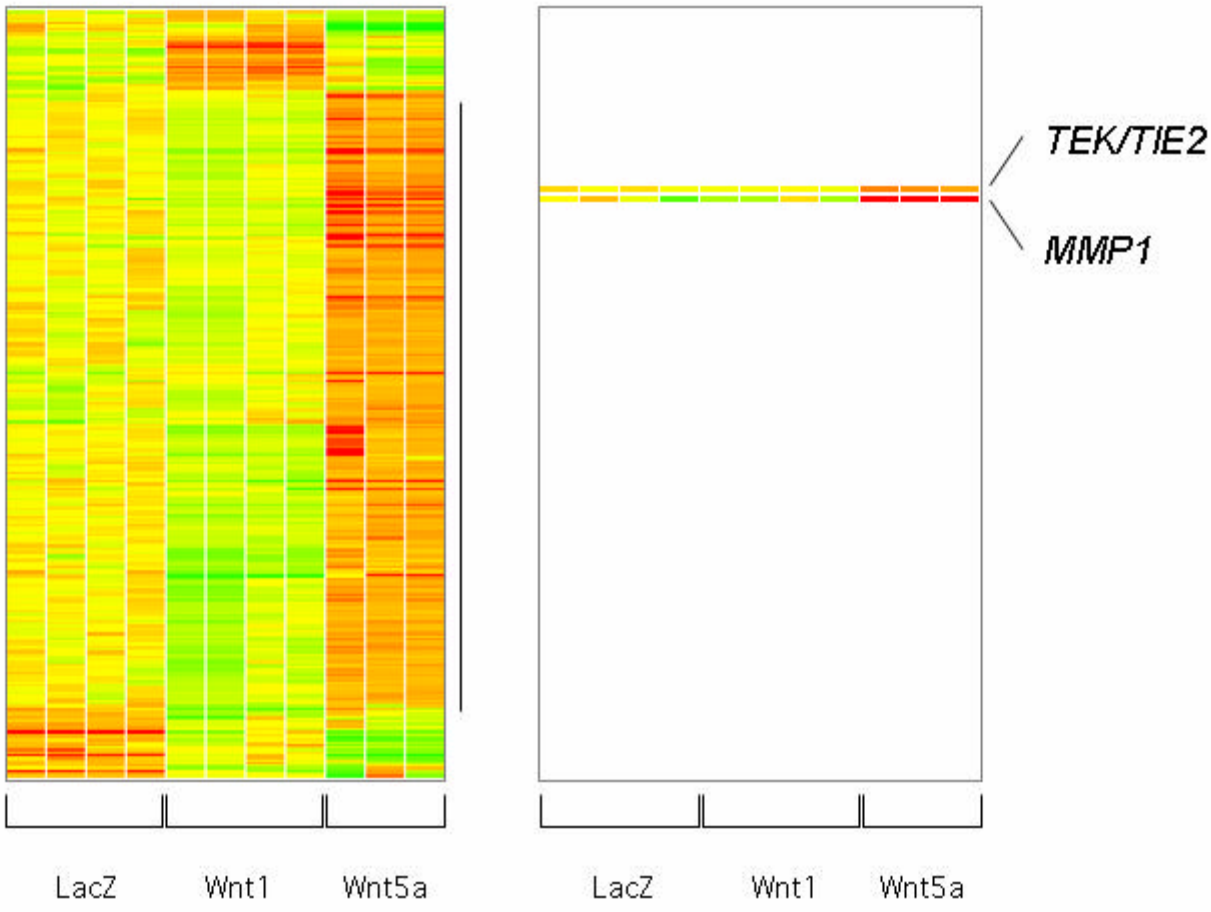

Figure 3

DNA array analysis of HUVEC ectopically overexpressing LacZ (control), Wnt-1 or Wnt5a, after adenoviral infection. Data analysis produced a set of 297 probe sets, which were subjected to hierarchical clustering. Of these, 239 probe sets were in the well-defined clade (vertical line) with the highest expression in Wnt5a expressing cells, lowest expression in Wnt-1 cells and intermediate expression in LacZ. Colors represent a range of gene expression: low (green), intermediate (yellow) and high (red) relative to experiment mean.

Symbol	Annotations	Fold
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	245.0
APOE	Apolipoprotein E	22.4
ELN	Elastin (supravalvular aortic stenosis, Williams-Beuren syndrome)	13.0
CDC25B	Cell division cycle 25B	5.7
PLCG2	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	5.2
RBP1	Retinol binding protein 1, cellular	2.9
ARHGAP4	Rho GTPase activating protein 4	2.7
TEK	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	2.3
PEPD	Peptidase D	2.1
NOS3	Nitric oxide synthase 3 (endothelial cell)	2.1

TABLE I

Table I

DNA array analysis of endothelial cells ectopically overexpressing Wnt-5a after adenoviral infection. Shown are some of the genes selected with the highest expression in Wnt5a expressing cells, lowest expression in Wnt-1 cells and intermediate expression in LacZ. Genes are listed according to their fold change compared to LacZ control infection.

FIGURE 4

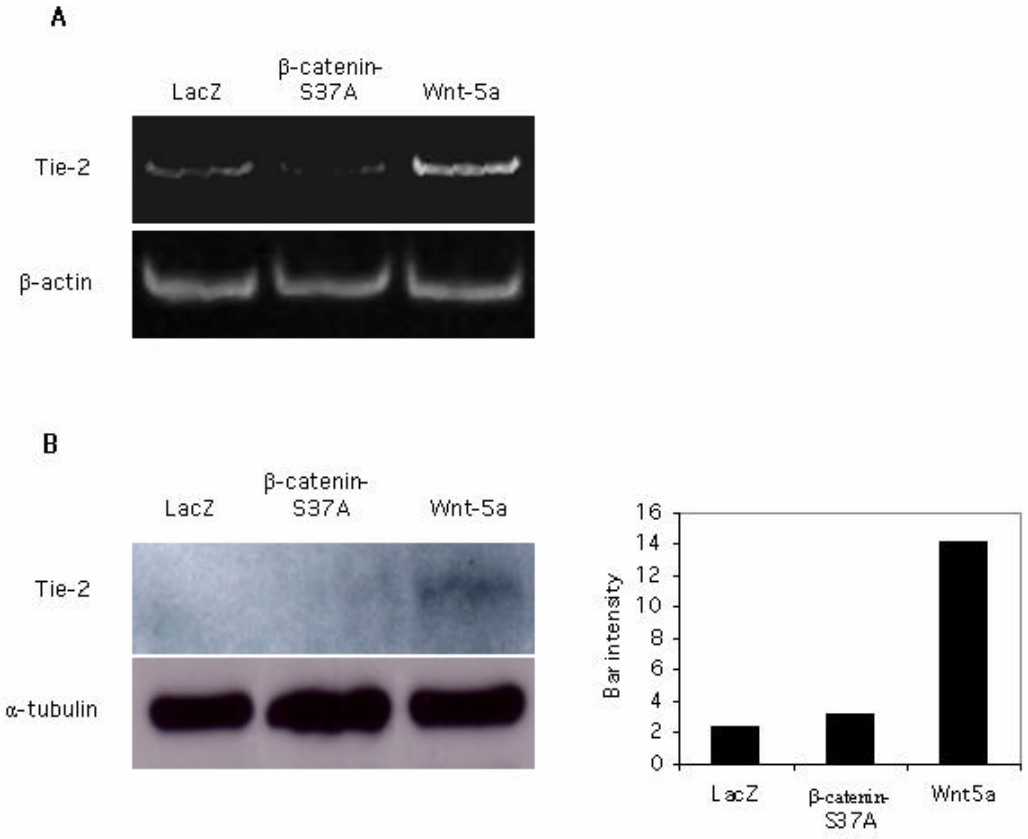

Figure 4

Wnt5a induces expression of Tie-2 in HMVEC. Ectopic expression of control LacZ, ß-cateninS37A or Wnt5a gene was performed by adenoviral infections. (A) Tie-2 expression analysis by RT-PCR. ß-actin was used to normalize samples. (B) Western blot analysis using an anti-Tie2 antibody on plasma membrane proteins purified after surface biotinylation of cells. a-tubulin Western blot was performed from cell lysates to normalize protein content.

Key research accomplishments

During the last period of this project, highly significant findings were achieved in showing that non-canonical Wnt signaling plays an active role in angiogenesis. These findings are summarized below under "Conclusions". New insights into intracellular responses to non-canonical Wnt signaling were found. After finding an effective sequence to silence the human Wnt5a gene, new *in vitro* effects of Wnt5a signaling on endothelial cell biology were detected, including reduced network formation and cell migration. Additionally, using DNA array data obtained during the previous year, a new target gene regulated by non-canonical Wnt signaling was validated.

Reportable Outcomes

During the last year, experimental work has been performed accoring to the guidelines provided by the latest Statement ot Work (see Appendix 1). New results are currently in the process of being published in Molecular Biology of the Cell. At the time of writing the present report a large manuscript is accepted for publication with some revisions/corrections by this journal and will be resubmitted soon. In the last year, another publication was also accepted and published by a review journal (Physiology), attached in the appendices. A book chapter entitled "Wnts in angiogenesis" is currently in the final steps of preparation. This chapter will be part of a volume of Advances in Developmental Biology entirely dedicated to Wnt signaling which will be published in 2007. Also, one more manuscript is being experimentally completed and prepared on Norrin, a new non-Wnt ligand of Frizzled-4, which has shown to be highly active in endothelial cell and retinal capillary function. Additionally, a Poster communication on non-canonical Wnt signaling in endothelial cells was presented at the International Wnt Meeting in Aberdeen, Scotland in September 2005 (see Abstract in appendix 3). All the above mentioned publications and the presentation have the PI as first author and acknowledged or will acknowledge the DOD and the present award.

As part of collaboration work with other laboratories at Columbia University, two more publications on Wnt signaling are being prepared with the PI as a co-author (with second and fourth authorship). These manuscripts will be soon submitted to scientific journals with high impact factor.

Conclusions

For the last year of work in the project, experimental results allow to conclude that:

- Wnt5a can induce phosphorylation of Dishevelled2 and Dishevelled3, two crucial mediators of intracellular Wnt signaling, in HUVEC.
- ERK1/2, an important regulator of proliferation and other cell functions, can be phosphorylated in HUVEC after Wnt5a induction.
- Non-canonical Wnt signaling can antagonize canonical signaling in HUVEC.

- A role for non-canonical Wnt signaling in endothelial cell network formation was confirmed. Consistent with this finding, and after discovering an effective target sequence to silence the human Wnt5a gene with shRNA technology, Wnt5a silencing impaired endothelial cell network formation in vitro.
- Wnt5a expression silencing impaired wound migration of HUVEC in vitro. This evidences that non-canonical Wnt signaling can play a role in endothelial cell migration.
- Tie2, a crucial regulator of angiogenesis, was validated as a new target gene upregulated by Wnt5a.

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Part of the performance of experimental work and result analysis for the current project was done in colaboration with researchers from the PI´s and other laboratories. I would like to acknowledge Audrey Ahn, Nancy L. Parmalee, Chi-Ming Li, Alan Khoo, Benjamin Tycko, Anthony M.C. Brow and my advisor, Jan Kitajewski.

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Appendix 1

MASCKAUCHAN, TULIO N.H. LOG NUMBER: BC020712

STATEMENT OF WORK

"Activation of alternative Wnt signaling pathways in human mammary gland and breast cancer cells".

Task 1. To evaluate in vitro changes in the activity of Rho-GTPases and JNK in human mammary gland and breast cancer cell lines.

a. To develop and purify plasmids for expressing Wnts, Frizzled receptors, dishevelleds, axin, Rho-GTPases and JNK in mammalian cells (months 1- 4).

b. Set up of assays for Rho-GTPases and JNK and of transfections into a variety of human immortalized epithelial mammary gland cells and breast cancer cell lines (months 5 - 9).

c. Screening of activity for Rho-GTPases and JNK in the previously tested cells (months 10 - 19).

d. Analysis of biological effects of alternative/non-canonical Wnt-signaling in human endothelial cells in culture by overexpression of Wnts (months 20 – 26).

Task 2. Analysis of gene expression in response to induction of non-canonical Wnt signaling.

a. Development of DNA array analysis to screen for target genes regulated by non-canonical Wnt signaling (months 27 - 28).

b. Validation (RT-PCR and Western blots/activity assays) of selected genes detected by the previous DNA array analysis (months 29 - 30).

c. Development and use of shRNA probes to interfere with alternative/non-canonical Wnt signaling in human cells and analysis of biological effects in vitro (months 31 -33).

d. Analysis of the interactions between non-canonical and canonical Wnt signaling (months 34 – 36).

Appendix 2

Materials and Methods

Cells and reagents

HUVEC were isolated from human umbilical vein as previously described (Jaffe et al, 1973) and grown in EGM2 (BioWhittaker) with VEGF, bFGF, EGF, IGF-1 and 2% FBS on dishes coated with Type I rat tail collagen (Upstate Biotechnology, USA). HMVEC and their media were obtained from BioWhittaker, Inc. (USA). Adenoviruses encoding for Wnts or mutant ß-catenin were prepared as described (Young et al, 2003). SuperTopFlash and SuperFopFlash Tcf luciferase reporter constructs were generously provided by Dr. Randall Moon (University of Washington School of Medicine, Seattle, WA). Purified Wnt5a protein was obtained from R&D Systems Inc. USA. Antibodies were used to recognize human ß-catenin (BD Transduction Laboratories, USA), Dishevelled2 or Dishevelled3 (Santa Cruz Biotechnology, USA), MMP-1 and Tie2 (R&D Systems Inc, USA), p44/p42 (ERK1/2) (Cell Signalling Technology, Inc, USA) or a-tubulin antibody (Sigma-Aldrich Life Sciences, USA).

Gene transfer into HUVEC

To infect cells, $4x10^5$ passage 3-5 cells were trypsinized and resuspended in 300 µl full culture medium. Adenovirus stock was added at 30 MOI unless indicated otherwise for each experiment, and cells were incubated at 37°C for 1 hour with gentle shaking every 10 min. Then, cells were seeded onto type I collagen-coated plates and harvested 24-48 hours later depending on the assay. For retroviral gene transfer, the retroviral vector pHyTCX was used. $5.0x10^6$ GP293 packaging cells (Clontech) were seeded, transfected with 10 µg pHyTC-genes and pVSVG and retroviral-containing supernatants were collected 48 hr later. Retroviruses were added to passage 4 HUVEC and 48 hrs later cells were selected for 4-5 days with hygromycinB at 300 µg/ml and then maintained with hygromycinB at 100 µg/ml. An expression vector encoding the HA-tag (pHyTC-HA) was used as negative controls. Protein expression in HUVEC was evaluated by immunoblotting using antibodies to the HA epitope in order to proper expression of Wnt.

Wnt5a gene silencing

A series of 6 different target sequences in the human Wnt5a gene were chosen for screening of adequate silencing of this gene. Synthesized oligos were designed using these sequences and cloned into pSIREN retroviral vector (BD Biosciences, USA). Obtained plasmids were used to screen for successful silencing by transfecting 293 cells in culture. Levels of Wnt5a expression were evaluated after reverse transcription of the RNA samples followed by PCR using primers for Wnt5a in conditions described above. The target sense sequence selected was 5´ AGTGCAATGTCTTCCAAGT. Preparation of HUVEC retroviral lines was

performed as described above, and successful silencing of the Wnt5a gene was confirmed by semi-quantitative PCR.

Reporter assays

Cells were seeded on collagen-coated 24-well plates (33,000 cells per well). The next day, cells were transfected with either Tcf/Lef transcriptional activation reporter construct SuperTopFlash containing Tcf responsive elements or SuperFopFlash with mutated elements (control) and a renilla-luciferase construct. Transfections were performed in triplicates using 0.18 µg of reporter plasmid, 0.02 µg of renilla-luciferase plasmid and 0.45 µg of inducer plasmid in total with 1.3 µl of Lipofectin in OptiMEM media (Invitrogen, USA) per well, following manufacturer's instructions for procedure. Cells were incubated at 37°C for 5 hours with transfection cocktail and then were incubated overnight with fresh full endothelial culture media. Cell lysates were prepared the next day and both firefly and renilla luciferase activities were evaluated using Dual-Luciferase Reporter Assay System (Promega, USA). Samples were read in a luminometer and values were normalized for transfection efficiency using renilla-luciferase activity.

Endothelial network formation assay

Retroviral selected HUVEC expressing Wnt-5a-HA or control gene (HA tag) were analyzed by Western blot to confirm the overexpression of the desired protein. Matrigels were prepared using 24-well plates with 0.3 mL pure Growth Factor-reduced Matrigel (BD Biosciences) per well and incubating plates at 37°C for 1 hour. Retroviral selected HUVEC grown on collagen-coated plates were seeded at 100,000 cells per well on top of Matrigel in the presence of 0.8 mL of full EC culture medium. Pictures were taken with 4x magnification after 18-19 hours of incubation at 37°C. All experiments were performed in duplicates and repeated twice to confirm results.

RT-PCR and DNA array analysis

Low passage human primary endothelial cells were cultured in EGM-2 BulletKit (BioWhittaker, Inc.). Total RNA was isolated using RNeasy Protect kit (Qiagen, Germany), reverse transcription reaction was performed using Omniscript Reverse Transcriptase as described by manufacturer´s instructions (Qiagen, Germany) and PCR was done using Platinum Taq DNA Polymerase (Invitrogen, Inc.). Conditions for Wnt-5a and Wnt-5b PCR were 94°C 3 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72 °C for 1 min plus 2 min at 72 °C. Primers used for Tie2 were 5´-TAATGAGACAATGCTGGC forward, and 5´-CATGTTTTCTCAGCAGTTG reverse, with same conditions as above but annealing at 60°C and product size of 241 bp. To correct for sample variations in RT-PCR efficiency, ß-actin expression was used to normalize the RNA samples using the following primers: 5´-CGAGGCCCAGAGCAAGAGAG forward; 5´-CTCGTAGATGGGCACAG TGTG reverse with a product size of 336 bp. Conditions for ß-actin PCR were

94°C 2 min followed by 20 cycles of 94°C for 45 sec, 60°C for 1 min and 72 °C for 1 min plus 5 min at 72 °C. DNA array analysis was performed with cDNA obtained from HUVEC adeno-infected to express either Wnt-5a or LacZ gene (control) at 48 hours post-infection. Hu95Av2 GeneChips, which query about 10,000 genes, were purchased from Affymetrix (Santa Clara, CA). Microarray and probes were prepared and used as described before (Li et al, 2002). To anaylize data, for each sample, the signals for each gene (probe set) were normalized to the values of the entire microarray and statistical analyses were done using GeneSpring TM Software (Silicon Genetics).

Western Blots

Protein expression in HUVEC was evaluated by immunoblotting using a human ß-catenin antibody from mouse (BD Transduction Laboratories, CA), human MMP-1 antibody from mouse (R&D Systems Inc, MN) or an a-tubulin antibody (Sigma-Aldrich Life Sciences, MO) when corresponding. For the evaluation of cytosolic ß-catenin levels, HUVECs were infected with adenoviruses encoding for Wnt-1, Wnt-5a or control gene (LacZ) as described above and seeded on 10 cm diameter dishes. Cytosols were isolated 48 hrs later by first washing the cells with ice cold PBS three times and then adding 750 ul of physiological buffer (10 mM TRIS-HCl pH=7.4, 140 mM NaCl, 5 mM EDTA, 2 mM DTT, 0.5 mM PMSF, 0.2 µg/mL aprotinin, 0.1 mg/mL leupeptin) to each dish. Cells were scraped with a rubber policeman and lysed in a chilled Dounce homogeneizer. Lysates were cleared from unbroken cells and nuclei by centrifugation at 500g for 10 min and the supernatants were then centrifuged at approx. 100,000g for 90 min. Cytosolic fractions were frozen at -20°C until the protein content was evaluated with a human ß-catenin antibody. For the Western blot of MMP-1 and a-tubulin, lysates were prepared as indicated by the manufacturer of the corresponding antibody. Proteins were visualized by Enhanced Chemiluminescence (Amersham) in conjunction with goat anti-mouse IgG-HRP.

Appendix 3

"Non-canonical Wnt Signaling Promotes Proliferation and Expression of Angiogenic Regulators in Human Endothelial Cells"

Masckauchán, T. Nestor H., and Kitajewski, Jan.*

Poster Presentation - International Wnt Meeting – Aberdeen, Scotland – September 2005.

Angiogenesis, the process of developing new vessels by sprouting from existing ones, is essential in embryonic development as well as in the growth of many types of tumors. Our research focuses on the biological effects of Wnt-5a, a non-canonical Wnt, on primary human endothelial cells in vitro, as well as the mechanisms underlying those effects. We observed that activation of non-canonical Wnt signaling by Wnt-5a can regulate proliferation of endothelial cells in vitro. We tested endothelial cells infected with adenoviruses encoding for Wnt-5a incubated in presence of basal medium added with FGF. Cells overexpressing Wnt-5a were able to proliferate faster, as well as showed an enhanced survival when they were cultured at confluency under the same conditions. Similar results were obtained by incubating cells with purified Wnt-5a protein. Expression of an interfering shWnt-5a construct led to reduced growth rate of HUVEC (human umbilical vein endothelial cells) when compared to control cells. Retrovirally-infected endothelial cells overexpressing Wnt-5a were able to induce better networks of endothelial cells on top of Matrigels. Consistent with this, HUVEC expressing shWnt-5a by retroviral infection showed a reduced ability to form networks under the same conditions. The Frizzled-4 extracellular domain (ECD) can at least partially block the Wnt-5a induction of proliferation, while Frizzled-5 and Frizzled-2 ECDs were unable to, indicating that Frizzled-4 is the receptor that mediates the proliferative response to Wnt-5a in human endothelial cells. As Wnt-5a is capable of negatively regulating canonical Wnt signaling in HUVEC, we hypothesize that both canonical and non-canonical Wnt signaling can regulate angiogenesis through different and independent mechanisms in human primary endothelial cells. Screening for Wnt-5a regulated genes in endothelial cells with a DNA array chip, that includes over 10,000 human genes, identified matrix metalloproteinase-1 (MMP1) and Tie2 receptor as Wnt-5a-regulated genes. Upregulation of these two genes by Wnt-5a overexpression were validated by RT-PCR and Western blot data. Both genes are implicated in the angiogenic process and may be involved in the Wnt-5a angiogenic function.

Appendix 4 – Publications

Masckauchan TN and Kitajewski J. Wnt/Frizzled Signaling in the Vasculature: New Angiogenic Factors in Sight. Physiology 21:181-188, 2006.

Masckauchán, TNH; Agalliu, D; Vorontchikhina M; Ahn A; Parmalee NL; Li CM; Khoo A.; Tycko B, Brown AMC Kitajewski J. Wnt5a Signaling Induces Proliferation and Survival of Endothelial Cells and Expression of MMP-1 and Tie2. (Accepted with corrections – to be resubmitted to Mol. Biol. Cell).

PHYSIOLOGY 21: 181–188, 2006; 10.1152/physiol.00058.2005

Wnt/Frizzled Signaling in the Vasculature: New Angiogenic Factors in Sight

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Wnt growth factors function via Frizzled receptors to affect cellular proliferation, differentiation, apoptosis, and migration. Wnt/Frizzled signaling is now linked to human hereditary disorders with retinal vascular defects, implicating Wnts as angiogenic factors. Here, we discuss Wnts and a novel Frizzled ligand, Norrin, in physiological and pathological angiogenesis.

Wnts are secreted, cysteine-rich glycoproteins that bind and activate Frizzled receptors, a family of seven transmembrane domain proteins. Current knowledge of Wnt signaling has been garnered from a variety of organisms, including mouse, fly, zebrafish, *Xenopus laevis*, and using mammalian cultured cells (73). Wnts govern cell proliferation, survival, differentiation, polarization, and migration by modulating both cellular and transcriptional events (49). Based on studies in mice, Wnts function during mammalian development, but Wnt signaling is also commonly altered in human cancers. The signaling cascades defined for Wnt/Frizzleds are uniquely distinct from other receptor-mediated pathways, but their diversity of cellular outputs still presents a challenge to our understanding of how Wnts work. Cellular activities governed by Wnts are also those that are critical for vascular development and angiogenesis, the process by which new blood vessels sprout from preexisting ones. Thus one might expect that Wnts would be implicated as angiogenic factors. The focus of this review is on recent studies exploring the hypothesis that Wnts are angiogenic factors and on the discovery of human retinal vascular disorders associated with Frizzleds. We will briefly overview the mechanisms of intracellular Wnt signaling, evaluate genetic and biochemical evidence that Wnts act in vascular development, and describe a novel Frizzled ligand, Norrin. These studies place Wnt/Frizzled signaling central to the development of retinal vasculature. The evidence that Wnt/Frizzled represents a novel angiogenic signaling pathway is now in sight.

Wnts and Frizzleds: Canonical and Noncanonical Wnt Signaling Pathways

Wnts signal through Frizzleds and the transduction of the signal has been separated into a "canonical" pathway and several "noncanonical" pathways (FIGURE 1) (reviewed in Refs. 49, 54, 73). Canonical Wnt signaling involves stabilization of cytosolic β-catenin, turning it into a nuclear transcriptional regulator. Noncanonical signaling represents several signaling cascades activated by Wnt/Frizzleds that do not work via β-catenin. Both canonical and noncanonical signaling work through Frizzled activation, which involves recruitment of the cytosolic Dishevelled proteins to the intracellular domain of Frizzled. After this event, the canonical and noncanonical pathways diverge in their mechanisms of action. Frizzleds contain seven transmembrane domains, an extracellular cysteine-rich domain (CRD) necessary for binding to Wnts, and an intracellular domain containing a conserved motif for Dishevelled binding (69). The low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) function as Frizzled co-receptors in the canonical pathway, forming a ternary signaling complex on binding of Wnt to Frizzled (64).

During the resting state of canonical Wnt signaling, several cytosolic proteins form a large complex to reduce the amount of cytosolic β-catenin via proteolysis. This complex includes the tumor suppressor adenomatous polyposis coli (APC), which mediates the targeting of β-catenin to the ubiquitin-mediated proteolysis pathway. Also participating in this complex is Axin, a scaffold that brings the enzyme glycogen synthase kinase 3 and cytosolic β-catenin in proximity leading to phosphorylation of β-catenin. Activation of the canonical pathway by a Wnt results in inhibition of this turnover complex leading to increased cytosolic β-catenin, which then transits to the nucleus. In the nucleus, β-catenin associates with one of a family of Tcf/Lef transcription factors (4). Tcf/β-catenin complexes directly regulate the expression of numerous known target genes, many of which, such as cyclinD1 (65) and c-myc (24), are implicated in cellular proliferation. We will refer to this pathway as Wnt/β-catenin signaling.

Noncanonical Wnt signaling can mediate proliferation (76) and cellular movements during gastrulation in vertebrates (35) and regulate planar cell polarity (PCP) in both vertebrates (21) and invertebrates (47). Intracellular transmission of noncanonical Wnt signaling utilizes Dishevelled (2); however, there are several distinct branches of noncanonical signaling. The Wnt/PCP branch of noncanonical signaling is known to act through Rho-GTPases and JNK to regulate orientation of cellular structures (47). The Wnt/Ca^{2+} pathway utilizes G proteins or intracellular calcium influx with activation of calcium-sensitive kinases PKC and Ca^{2+}/calmodulin-dependent protein kinase II to effect Wnt-mediated signals (36). Research is still ongoing as to the function of the distinct noncanonical signaling

branches and their roles in regulating different biological phenomena. The two forms of intracellular Wnt signaling, canonical and noncanonical, conduct signaling cross talk. For instance, noncanonical Wnt signaling can antagonize Wnt/β-catenin signaling, a phenomenon that may occur through several mechanisms (29, 68, 70).

Wnt Signaling Pathways and Vascular Biology

Vascular development depends on Wnt signaling, as evidenced by genetic analysis of embryos with mutations in Wnt or Frizzled genes.

Wnt-2-deficient mouse embryos fail to establish a proper fetal capillary network in the placenta (48). Consistent with this finding is the observation that Wnt-2 is expressed in fetal vessels of the placenta (48). Thus Wnt-2, which can activate Wnt/β-catenin signaling (60), is implicated in the consolidation of proper placental vascularization.

Vascular formation in the mammalian gonad occurs in a sex-specific manner, during which endothelial cells migrate from the mesonephros into the gonad to form a coelomic blood vessel. Analysis of Wnt-4 knockout mice showed that Wnt-4 represses mesonephric endothelial migration in the XX gonad, preventing the formation of male-specific coelomic blood vessels (30).

The Wnt-7b gene functions in lung vascular development, as Wnt-7b embryos bearing a gene replacement of the first exon of the Wnt-7b gene with the *lacZ* gene (Wnt-7b$^{LacZ-/-}$) display severe defects in the smooth muscle component of the major pulmonary vessels (62). Mutant embryos at E18.5 had enlarged, branched vessels surrounded by extensive hemorrhage in the lungs, whereas increased cell death in vascular smooth muscle cells was detected in mutant neonates. Thus loss of Wnt-7b function results in loss of vascular smooth muscle integrity leading to pulmonary hemorrhage. Wnt-7b signals through Frizzled-1 and Frizzled-10, in cooperation with LRP5, to activate Wnt/β-catenin signaling (72). However, a different phenotype is associated with a Wnt-7b knockout mouse that targets the third exon. This Wnt-7b deficiency caused impaired chorion-allantois fusion during placental development but no evident vascular defects (53). Wnt-7b has also been shown to be required for the initiation of apoptosis and regression of transient ocular hyaloid vessels in mice (39).

The Frizzled-5 gene is expressed in the yolk sac, eye, and lung bud in 9.5-day-old embryos, and loss of Frizzled-5 gene function leads to death around *day 11* of embryonic development due to severe defects in yolk

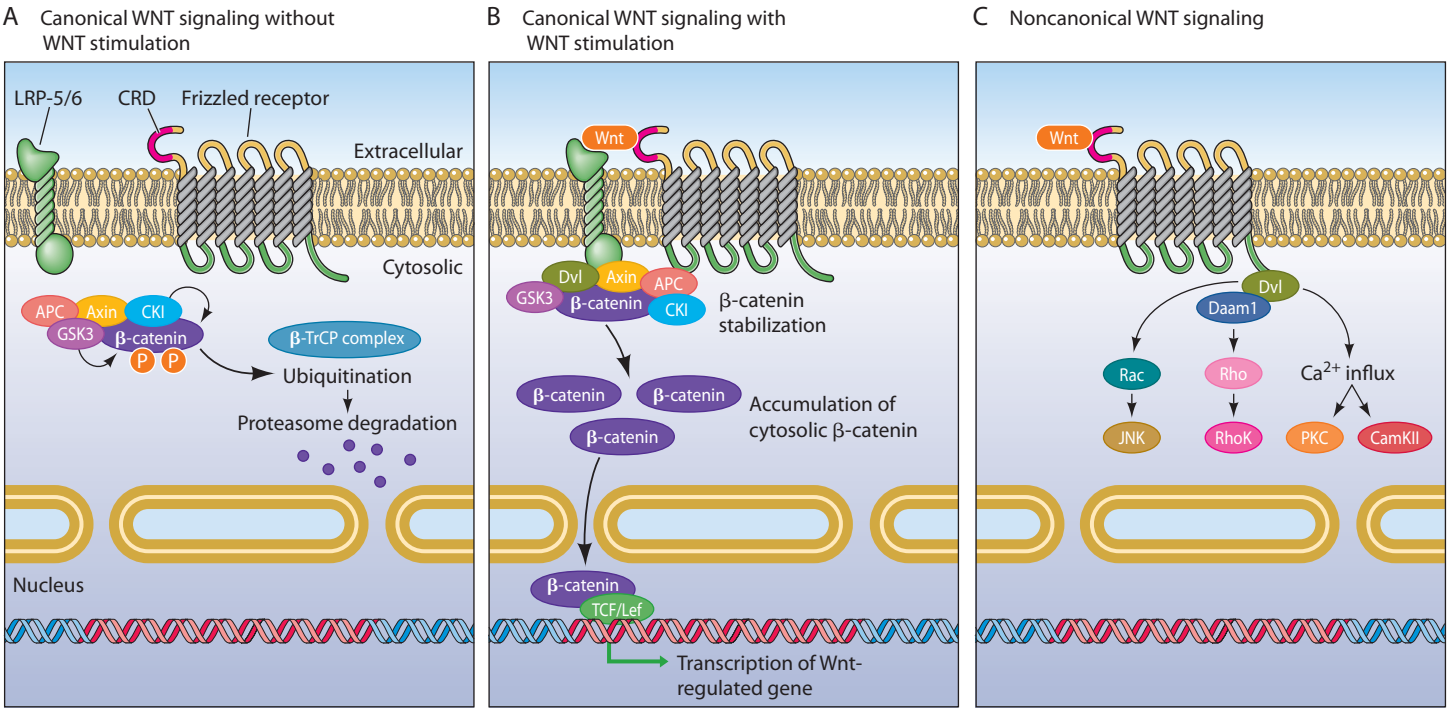

FIGURE 1. **Wnt signaling transduction pathway**
A: in the canonical Wnt pathway or Wnt/β-catenin pathway, when signaling is at the basal state (*top*), cytosolic β-catenin is phosphorylated by GSK3 and CKI in a multiprotein complex containing the scaffold protein Axin and APC. Phosphorylated β-catenin is then ubiquitinated by β-TrCP and degraded in proteasomes. In presence of a Wnt that activates Wnt/β-catenin signaling (*bottom*), Dishevelled (Dvl) blocks the β-catenin degradation complex, allowing its cytosolic accumulation. Stabilized β-catenin then translocates into the nucleus where it associates with TCF/Lef transcription factors, leading to regulation of numerous target genes. *B*: in the noncanonical Wnt pathway, Dvl is linked through Daam1 to allow activation of small GTPase Rho and Rho-kinase (RhoK) and can activate JNK via Rac. In *Drosophila*, this pathway can direct cytoskeletal organization and coordinated polarization of cells in an epithelial sheet. Wnts that function in noncanonical signaling can also induce intracellular calcium flux and the activation of calcium-sensitive enzymes such as PKC and CamKII. As several other players participate in these cascades, simplified versions are schematized.

sac angiogenesis (28). Lack of Frizzled-5 reduces endothelial cell proliferation in yolk sac vasculature and, later in development, disrupts placental vasculogenesis.

Which cell types of the vasculature participate in Wnt signaling? Wnts are paracrine factors and may be produced by endothelial, mural, or epithelial cells acting on Frizzleds found on endothelial cells. Thus Wnts could conceivably be expressed from a variety of cellular sources and affect endothelial cells. Wnts and Frizzleds are endogenously expressed in both endothelial and vascular smooth muscle cells. One study reported that Wnt-5a and Frizzled-3 are expressed in human umbilical vein endothelial cells (HUVEC) and smooth muscle cells from human pulmonary artery, whereas mouse brain microvascular cells showed expression of Wnt-7a and Wnt-10b and Frizzled-1 (74). The Wnt-7a, Wnt-10b, and Wnt-13 genes and Frizzled-4, Frizzled-5, and Frizzled-6 genes are expressed in HUVEC and human dermal microvascular cells (HMVEC) (14, 44). Human umbilical vein and microvascular endothelial cells also express β-catenin-associated transcription factors Tcf-1, Tcf-3, Tcf-4, and Lef-1 (44). Of the Frizzleds expressed in cultured endothelial cells, Frizzled-4 and Frizzled-5 are linked to vascular development by genetic analysis in either mouse (Frizzled-5) or humans (Frizzled-4). β-Catenin signaling occurs in endothelial cells in vivo, as defined by following T-cell factor (TCF) transcriptional activity in whole animals. A β-catenin-activated transgenic (BAT) mouse driving expression of nuclear β-galactosidase reporter (BAT-gal) has been made that expresses lacZ under the control of β-catenin/TCF responsive elements. BAT-gal expression identifies a variety of sites of Wnt signaling, like notochord and brain, but also identifies endothelial cells as a site of β-catenin/TCF signaling (43).

Ectopic expression of Wnts in cultured endothelial cells can elicit biological responses, allowing a better understanding of the precise role Wnts play in the steps of angiogenesis. Pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), promote endothelial survival, proliferation, migration, and morphogenesis, and regulate the association of endothelial cells with the extracellular matrix and mural cells (20). These distinct cellular steps in angiogenesis have been evaluated in cultured endothelial cells undergoing Wnt signal activation, typically via ectopic expression of a Wnt. Mouse brain microvascular endothelial cells showed increased proliferation when Wnt-1, a canonical Wnt, was ectopically overexpressed (74). This observation was later extended to overexpressed mutant forms of β-catenin in a human microvascular endothelial cell line (71). Thus, as in other cell types, Wnt/β-catenin signaling in endothelial cells promotes proliferation. Angiogenic stimulation includes survival signals for endothelial cells, and Wnt-1/β-catenin signaling promotes the survival of human primary endothelial cells when cultured in low serum (44). Activation of Wnt/β-catenin signaling can also promote formation of capillary-like networks developed by primary endothelial cells in vitro, an activity that may reflect Wnt as either a morphogenic or survival factor (44).

The action of Wnts that signal via β-catenin as angiogenic factors may occur through indirect means. Seven β-catenin/TCF binding sites occur in the gene promoter for VEGF-A (18). VEGF is upregulated as a result of mutational activation of Wnt/β-catenin signaling in colon cancer cells (18, 77) and in human endothelial cells (63). Gene expression changes regulated by Wnt signaling in human endothelial cells led to the identification of the interleukin-8 gene as a target of Wnt/β-catenin signaling in endothelial cells (44), as in other cell types (37). Interleukin-8 can induce endothelial cell proliferation, survival, and in vitro expression of MMP-2 and MMP-9, two matrix metalloproteinases that function in angiogenesis (38). Thus Wnt/β-catenin may influence endothelial cells directly or indirectly via the induction of known angiogenic factors such as VEGF and IL-8, particularly during tumor angiogenesis. A variety of other angiogenic regulators have previously been reported as Wnt target genes (Table 1), including Eph/Eprhins (3), FGF18 (61), FGF20 (11), endothelin-1 (33), Cx43 (1), uPAR (42), MMP7 (9, 15), and MMP3 (55). Thus Wnts may regulate angiogenesis through induction of multiple angiogenic genes.

Stimulation of endothelial proliferation by β-catenin may also be mediated by platelet endothelial cell adhesion modecule (PECAM) signaling (6). Wnt/β-catenin activity is implicated downstream of several distinct signaling pathways that function in endothelial cells (22, 26). Changes in localization of β-catenin from the membrane to the cytosol, an indicator of Wnt/β-catenin signaling, is found in endothelial cells during neovascularization after experimental-induced myocardial infarction (7). However, conditional inactivation of the β-catenin gene in endothelial cells caused increased vascular fragility during embryogenesis, likely due to an alteration of catenin/cadherin function (10).

Secreted Frizzled-related proteins (sFRPs) are a group of secreted proteins with structural homology to the extracellular CRD of Frizzled receptors. FrzA or sFRP-1, originally isolated from bovine aortic endothelium but also expressed in many other tissues, can reduce the proliferation of endothelial cells in vitro (17). Recombinant, purified FrzA protein can temporarily arrest growth of endothelial and smooth muscle cells in vitro, increasing cytosolic levels of phosphorylated β-catenin and decreasing levels of cyclin E and cdk2 kinase while negatively controlling angiogenesis in an ischemic muscle in vivo model (19). However, in chick chorioallantoic membrane and in grafted mesenchymal and glioma cells, FrzA was able to induce formation of vessels while inducing migration and tube formation of endothelial cells in vitro (16). This apparent contradiction highlights the

fact that the role of FrzA in vascular biology is not well understood.

Wnt Signaling Comes into Play in Human Vascular Diseases

Impaired activity of Wnt signaling components have previously been directly linked to several human disorders, including colon and other cancers, fibrosis, tooth agenesis, and a rare disease characterized by complete absence of all four limbs called tetra-amelia (50). A direct link between Wnt signaling and a vascular phenotype in a human disorder was first reported as a connection between familial exudative vitreoretinopathy (FEVR) and mutations in the human FRIZZLED-4 gene (59). FEVR is a hereditary ocular disorder that develops with characteristic defective retinal vascularization combined with retinal detachments and leaky vasculature that bleeds and exudes. FEVR patients show a range of severity of these symptoms, with the most severe leading to blindness (41). FEVR is a genetically heterogeneous disorder, and so far four different FEVR loci have been mapped on several chromosomes, including 11q, 11p, and Xp (67). Mutations on the X chromosome linked to FEVR mapped to a novel gene referred to as the Norrie gene, sometimes referred to as the Norrie disease product (NDP) gene (12). The Norrie gene encodes for a protein product usually called Norrin and sometimes referred to as NDP, we will refer to the protein as Norrin. FRIZZLED-4 gene mutations (FIGURE 2) suggest that Wnt signaling is aberrant in FEVR. Frizzled-4 has been demonstrated to function in noncanonical Wnt signaling response in *X. laevis* embryos, since Frizzled-4 can mediate PKC translocation to the plasma membrane and induction of Ca^{2+}-/calmodulin-dependent protein kinase II (59). Experimental evidence demonstrates that Norrin can bind Frizzled-4 and trigger Wnt/β-catenin signaling (75). Thus Frizzled-4 is thought to function in both Wnt/Ca^{2+} and Wnt/β-catenin signaling. As support for Wnt signaling being important to FEVR, several FRIZZLED-4 mutant genes found in FEVR patients encode proteins that have reduced capacity to participate in noncanonical signaling (59). Several Frizzled-4 mutants were able to oligomerize with wild-type Frizzled-4, and this complex of mutant and wild-type Frizzled-4 was retained in the endoplasmic reticulum. Thus the genetic dominance of mutant Frizzled-4 in FEVR may reflect its ability to associate with wild-type Frizzled-4, blocking the capacity of endogenous Frizzled-4 to activate Wnt/β-catenin signaling (32).

The FRIZZLED-4 and the NDP genes lie at two of the four clearly identified FEVR loci. Thus other genes are also implicated in this disease. Mutations in the LRP5 gene (FIGURE 2), a known Wnt coreceptor, were described in some FEVR patients in a locus closely linked to the FRIZZLED-4 gene. Thus some mutations in the 11p region implicated in FEVR are found in the FRIZZLED-4 coding sequence and others are found in the LRP5 coding sequence. This implies that defective signaling can occur via production of mutant Frizzled-4 or LRP5 proteins (66). The identity of the genes mutated in two other FEVR loci are not known, but one may speculate that they encode players, novel or known, in Wnt signaling. It is currently estimated that mutations in one of five distinct genes can lead to

Table 1. Target genes induced by Wnt/β-catenin signaling

Target Gene	Refs.	Description	Function
EphB2/EphB3/Ephrin-B1	3	Receptors for ephrins and ephrin ligand	Vascular remodeling: cell-cell communication
VEGF	74	Vascular endothelial growth factor	Endothelial cell permeability, network formation: vascular permeability
FGF	11, 59	Fibroblast growth factor	Regulator of angiogenesis
EDN-1	32	Endothelin-1	Vascular cell manteinance
IL-8	36, 42	Interleukin-8	Endothelial cell proliferation and survival, network formation, regulation of MMPs
Cx43	1	Connexin43	Gap-junction communication: vasculogenesis and vascular remodeling
uPAR	40	Urokinase-type plasminogen activator receptor	Regulation of endothelial cell migration
MMP7	9, 15	Matrix metalloproteinase-7	Degradation of extracellular matrix: cell migration
MMP3	53	Matrix metalloproteinase-3	Degradation of extracellular matrix: cell migration

Target genes induced by Wnt/β-catenin signaling that encode known angiogenic regulators. The target genes were drawn from a comprehensive list provided by the Wnt homepage at http://www.stanford.edu/~rnusse/wntwindow.html.

manifestation of the FEVR phenotype in humans (67).

In agreement with the defects observed in FEVR, retinas from neonatal Frizzled-4$^{-/-}$ mutant mice were found to be completely devoid of the two intraretinal capillary beds (75). The Frizzled-4 mutant phenotype includes enlarged and tortuous major arteries and veins of the retina, and arteriolar arborization is diminished. Additionally, the nerve fiber layer shows a higher number of small fenestrated vessels at the vitreal face of the retina, which extend perpendicularly toward the inner retina. In the normal mouse, the hyaloid vasculature represents a set of vessels existing after birth in the vitreous body that completely regress by P17. The hyaloid vasculature is still present at the same stage in Frizzled-4$^{-/-}$ mouse, and retinal hemorrhages are common in these mice. Our studies demonstrate that Frizzled-4 is expressed in adult murine retinal vasculature (FIGURE 3).

Norrin, a New Non-Wnt Ligand of Frizzled

As its name suggests, the NDP gene has more commonly been associated with a human disorder termed Norrie disease (ND). Genetic linkage studies localized the NDP gene to the short arm of the X chromosome (8), and this led to the cloning and characterization of the NDP gene (5, 13). ND is an X-linked congenital retinal dysplasia involving blindness at birth and, sometimes, deafness and mental retardation. Originally described by Heine and Norrie in the 1930s (50a) and intensively studied by Warburg in the 1960s (72a, 72b), ND involves retinal detachment and retinal folds, vitreous opacity, and other symptoms that make it clinically similar to FEVR, except that ND can be associated with mental retardation and deafness.

Norrin, the protein encoded by the NDP gene, is a cystein-rich secreted product that belongs to the superfamily of growth factors containing a cysteine knot motif (45). Other members of the cysteine knot superfamily are transforming growth factor-β (TGF-β) and nerve growth factor (NGF). Genetic analysis in ND patients has revealed a wide variety of missense, nonsense, deletion, and splice-site mutations in the NDP gene. The reported mutations in ND patients number in excess of 100, and different mutations in the Norrin gene have been reported in patients with FEVR, retinopathy of prematurity (ROP), and Coats' disease. A schematic diagram of most characterized point mutations in Norrin are shown in FIGURE 2. Most of the mutations in patients either affect a normal cysteine residue of Norrin or amino acids in the vicinity of cysteine residues. These cysteines are well preserved in the superfamily of proteins to which Norrin belongs and may be crucial in protein tertiary structure. Although Norrin has no sequence homology with Wnts, the high degree of similarity in vascular phenotypes between the Frizzled-4$^{-/-}$ mutant mouse and the defects reported for the Ndph$^{-/-}$ mutant mice (57, 58) led investigators to further analyze the hypothesis that

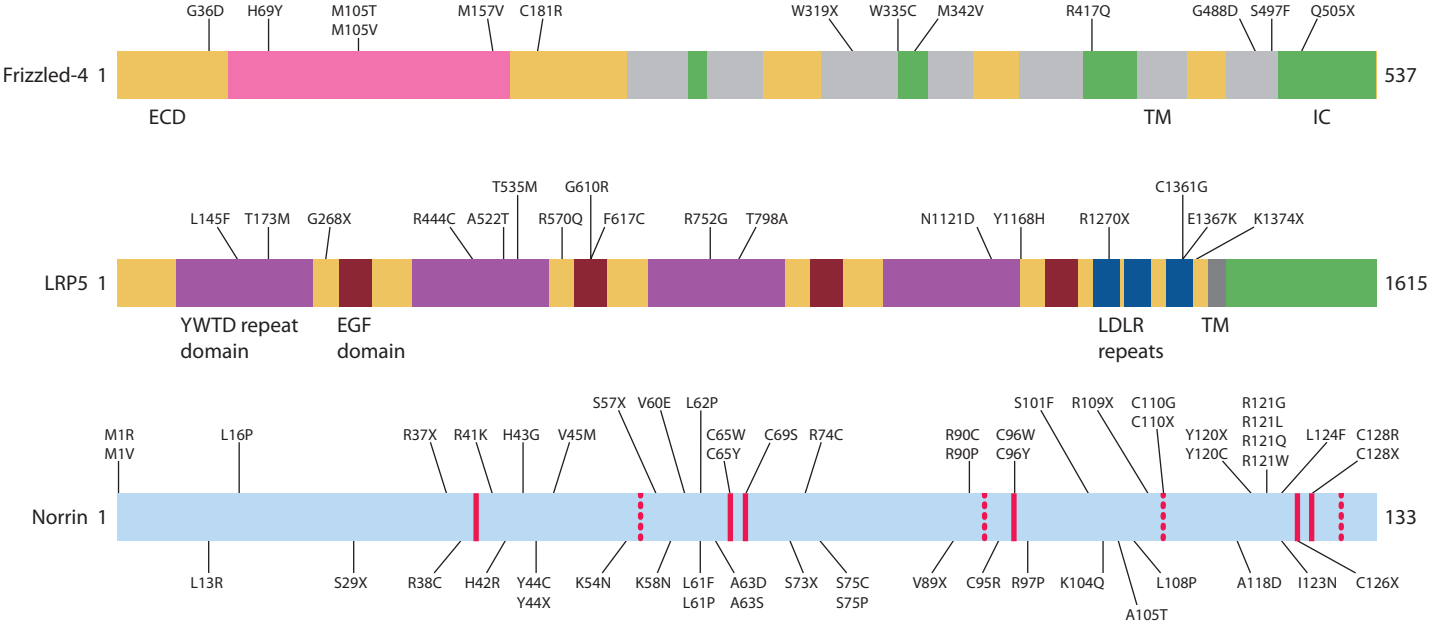

FIGURE 2. **Schematic diagram of Frizzled-4, LRP5, and Norrin proteins showing mutations within protein domains reported in ND, FEVR, ROP, or Coats' disease cases**

Only known nucleotide missense/nonsense substitutions were included. Transmembrane domains are depicted in gray, intracellular domains are in light green, and cysteine residues are represented by red lines. Extracellular domains (ECD) are represented in yellow. Frizzled-4: cystein-rich domain (CRD) domain is depicted as a pink color box, part of the full ECD. Transmembrane domains are represented as described previously (34). LRP5: the four portions of the ECD, including the five YWTD repeats (YWTD domain), are in purple, EGF domains are in dark brown, and LDLR repeats are in blue, with numbering as described in Ref. 25. Norrin: red dotted lines indicate position of cysteine residues involved in disulphide bridges. Other conserved cysteine residues in the cysteine knot domain are depicted in full red lines.

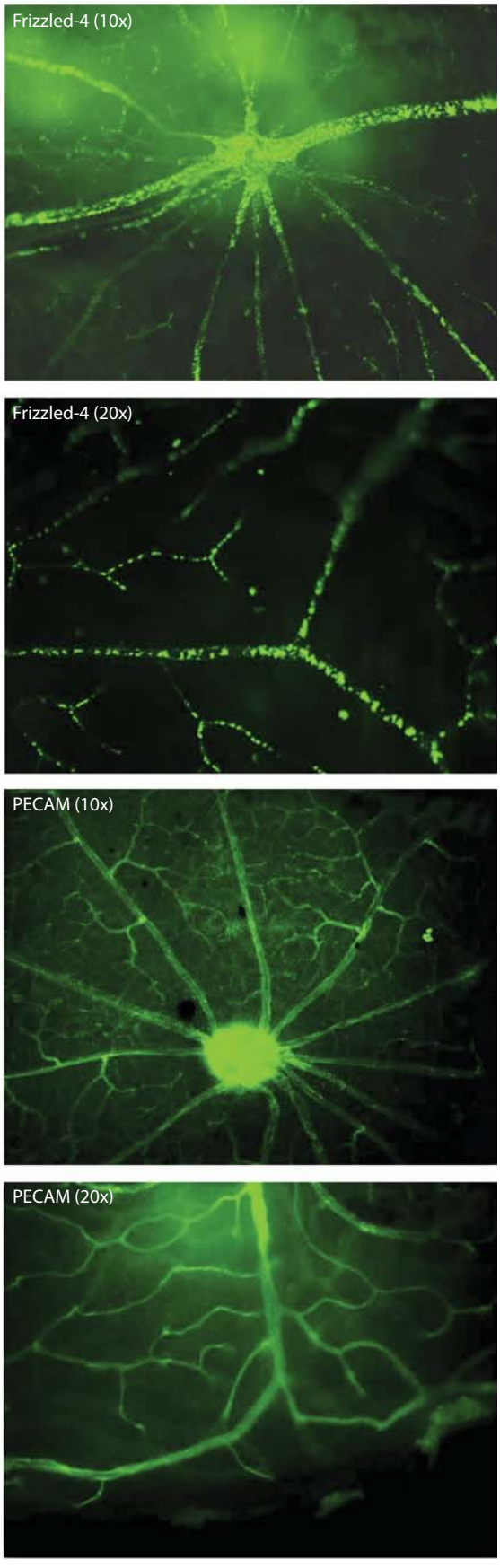

FIGURE 3. **Whole-mount immunofluorescence staining of adult mouse retinas for Frizzled-4 or PECAM, a known marker for endothelium**
Venal and arterial vasculature can be clearly seen (×10), as well as microcapillaries (×20), showing the presence of Frizzled-4 in vasculature of mouse retinas.

Norrin might be a Frizzled-4 ligand (75). Norrin showed high specificity of binding and affinity for Frizzled-4 in vitro. In addition, Norrin coexpression with Frizzled-4 led to activation of Wnt/β-catenin signaling in 293 cells, and several mutants of Norrin were unable to function in this assay. Thus, despite the lack of homology to Wnts, Norrin functions like a Wnt, binding the CRD domain of Frizzled-4 with nanomolar affinity and acting through a Frizzled and LRP coreceptor (73). Recently, it has been shown that Norrin is necessary for the proper regression of hyaloid vessels in mice after birth (52) as regression is delayed in mice expressing a truncated form of Norrin (58), as well as in $Lrp5^{-/-}$ mice (31) and Frizzled-$4^{-/-}$ mutant mice (75). Regression of hyaloid vasculature is partially mediated by macrophages, and a delay of hyaloid regression can affect subsequent retinal angiogenesis. The effects on retinal vasculature in FEVR or ND might thus be a direct consequence of impaired macrophage or hyaloid vascular cell function or secondary to hypoxia that develops. The defective retinal angiogenesis may also arise from a lack of normal functional Norrin in the outer retina alone or increased oxygen levels in the vitreous due to the extended presence of hyaloid vessels. These are important issues to resolve before proceeding with potential therapeutic intervention of the finely balanced development of hyaloid and retinal vessels.

Norrin has been initially described as a potential angiogenic factor for retinal vessels. Based on several observations, Norrin's function might not be strictly limited to the eye vasculature. Expression analysis showed significant levels of Norrin transcripts, not only in specific layers of the retina of human, mouse, and rabbit but also in cerebellum, hippocampus, olfactory bulb, and cortex of rabbit brain (23). An intriguing observation is the peripheral venous insufficiency reported in association with ND in a large Costa Rican family and in an isolated case in Great Britain (46, 56). That is, some Norrin mutations may lead to vascular defects in the venous system beyond the eye. Finally, homozygous mutation of the murine Norrin gene (Ndph) presents many features present in human ND. Loss of murine Norrin also leads to incomplete decidualization during pregnancy and defective vascular development of the decidua, leading to infertility (40). Thus there is a potential role for Norrin in female reproductive angiogenesis. Of note, a recently reported Frizzled-$4^{-/-}$ mutant mouse is also infertile, with nonfunctional corpora lutea probably due to impaired angiogenesis (27). The fact that, as opposed to Frizzled-$4^{-/-}$ mice, the $Ndph^{-/-}$ mice have functional corpora lutea indicates that Norrin, although expressed in the ovarian tissue, may not be the only mediator of Frizzled-4 signaling in the mouse ovaries. This opens the possibility of other angiogenic Frizzled-4 ligands yet to be determined. An important area for future study is to establish how widespread Frizzled-4 function as an angiogenic receptor beyond the retina.

Although therapeutic intervention in ND or FEVR is far from a reality, a light of hope is apparent based on Norrin. As proof of principle, a transgenic mouse model that ectopically expresses Norrin in Ndph$^{-/-}$ mutant mouse background partially restores the formation of normal retinal vascular network and retinal neuronal function. Norrin protein could similarly be applied or expressed in mutant eyes, setting the stage for evaluation of its therapeutic potential (51).

Concluding Remarks

The concept that Wnts and Norrin are a new class of angiogenic factor is gaining support. Clearly, Wnt/Frizzled signaling is critical for embryonic vascular development, and Norrin/Frizzled signaling is critical for angiogenesis in the developing eye. Whether angiogenic Wnt activities are mediated by Wnt/β-catenin or noncanonical Wnt signaling, or both, is still unresolved. Evidence exists for the involvement of both classes of Wnt signaling in angiogenesis. The first human developmental disorder linked to Wnt/Frizzled signaling was in fact FEVR, which was linked to Frizzled-4. The discovery of new FEVR loci may further implicate the Wnt pathway in retinal angiogenesis, and mouse modeling will likely be used to explore how prevalent Wnt/Frizzled signaling is in developmental and physiological angiogenesis. Because of these exciting discoveries, the field of Wnt/Norrin/Frizzled signaling in angiogenesis is now clearly visible. ■

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